Molecular Weight, Polydispersity, and Spectroscopic Properties of Aquatic Humic Substances

Yu-Ping Chin,*,† George Aiken,‡ and Edward O'Loughlin§

Department of Geological Sciences, The Ohio State University, Columbus, Ohio 43210-1002, Water Resources Division, U.S. Geological Survey, Boulder, Colorado 80303, and Environmental Sciences Program, The Ohio State University, Columbus, Ohio 43210

The number- and weight-averaged molecular weights of a number of aquatic fulvic acids, a commercial humic acid, and unfractionated organic matter from four natural water samples were measured by high-pressure size exclusion chromatography (HPSEC). Molecular weights determined in this manner compared favorably with those values reported in the literature. Both recent literature values and our data indicate that these substances are smaller and less polydisperse than previously believed. Moreover, the molecular weights of the organic matter from three of the four natural water samples compared favorably to the fulvic acid samples extracted from similar environments. Bulk spectroscopic properties of the fulvic substances such as molar absorptivity at 280 nm and the E_4/E_6 ratio were also measured. A strong correlation was observed between molar absorptivity, total aromaticity, and the weight average molecular weights of all the humic substances. This observation suggests that bulk spectroscopic properties can be used to quickly estimate the size of humic substances and their aromatic contents. Both parameters are important with respect to understanding humic substance mobility and their propensity to react with both organic and inorganic pollutants.

Introduction

Proper characterization of both the physical and chemical properties of humic substances or whole water organic matter is central toward understanding their role in the fate, reactivity, and transport of inorganic and organic pollutants (1–14). Measurement of the bulk properties of humic substances such as molecular weight, light absorptivity, and fluorescence can yield important information regarding their chemical reactivity and mobility (i.e., diffusivity). Many of these techniques use small sample volumes (microliter to milliliter) and are nondestructive. Unfortunately, these methods, if improperly executed, can lead to results that are artifactual and potentially misleading.

The measurement of molecular weights using size exclusion chromatography (SEC) has been criticized as a technique that is plagued by artifacts (15, 16). Indeed, humic substance molecular weights determined by other methods may differ from those measured by SEC by as much as 2 orders of magnitude (17–20). Recently, Chin and Gschwend (16) found that high-pressure size exclusion chromatography (HPSEC) can be used to measure reliably the molecular weights of humic and fulvic acids by the addition of an indifferent electrolyte (e.g., NaCl or KCl) to the mobile phase and by using random coil nonpro-

teinaceous polymers to calibrate the column. HPSEC-determined molecular weight distributions agreed reasonably well with Aldrich humic acid and Suwannee River fulvic acid literature values measured by vapor pressure osmometry (a measurement of analyte colligative properties), field flow fractionation, and ultracentrifugation (21–23). Thus, carefully designed and executed molecular weight measurements using HPSEC can result in weight- and number-averaged distributions that appear to corroborate reliably measured literature values for these two substances.

In this paper, we conducted a number of experiments that studied the spectroscopic properties and molecular size of dissolved and macromolecular organic materials from Lake Michigan sedimentary porewaters, a number of aquatic fulvic acids isolated from surface and subsurface waters from North America and Antarctica, surface water from the Suwannee River, and a commercial humic acid (Aldrich Chemicals). Our research objectives were to (a) compare the molecular weights for a large number of aquatic fulvic acids measured by HPSEC with those values determined by vapor pressure osmometry (VPO), low angle X-ray scattering, ultracentrifugation, and field flow fractionation; (b) study the spectroscopic properties of our isolated humic substances and unfractionated natural water organic matter; and (c) measure the molecular weight distributions of both the macromolecular and dissolved organic materials in whole-water samples from the Suwannee River and pore fluids separated from Lake Michigan sediments.

Methods

Preparation of Aquatic Fulvic and Humic Acid Solutions. Aquatic fulvic acids were isolated from different locations in the United States and Antarctica. These include the Suwannee River in Georgia; the Yakima River in Oregon (near Kiona); a Minnesota groundwater; the Ohio River (near Cincinnati, OH); Coal Creek, CO; Lake Fryxell in Antarctica; and the Missouri River (near Sioux City, IA). Commercial humic acid was obtained from Aldrich Chemicals. Porewater samples were collected by subcoring a boxcore sampled from the northern basin of Lake Michigan with a whole core squeezer. The squeezer mechanism forced the pore fluids through an acid-washed 70-μm Porex filter (Porex Technologies, Fairburn, GA) into a glass syringe. This procedure eliminates contact with the atmosphere and minimizes potential artifacts caused by other separation methods such as centrifugation (24). The syringes were allowed to stand, plunger down and partially covered with water, to settle out any large particles. Whole river-water samples were collected from the Suwannee River in Georgia and filtered through a 0.45-μm silver membrane filter.

Aquatic fulvic acids were isolated on Amberlite XAD-8 resin according to the method of Thurman and Malcolm

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* To whom correspondence should be addressed.
† Department of Geological Sciences, The Ohio State University.
‡ U.S. Geological Survey.
§ Environmental Sciences Program, The Ohio State University.

(25). For the purposes of the HPSEC and absorbance measurements, the isolated aquatic fulvic acid samples were dissolved in buffered water identical in composition to the HPSEC mobile phase (see below) at a concentration of 2.5 mg of fulvic acid in 25 mL of buffered water. This concentration yielded a total organic carbon (TOC) value of approximately 30–50 mg of C/L (depending upon the organic carbon content of the analyte), which was measured by organic carbon analysis (see below).

Aldrich humic acid solutions were treated extensively to remove the ash content, heavy metals, and residual fulvic acids. Details of this procedure can be found elsewhere (16). Aliquots from the stock solution were diluted in the mobile-phase buffer and filtered through a Gelman Type A/E glass fiber filter to remove any residual particulates. Porewaters from Lake Michigan were limited in sample size (only a few milliliters at best) and were used without any further pretreatment.

Molecular Weight Determinations. Number-averaged molecular weights of these isolates were determined by the vapor pressure osmometry method described by Aiken and Malcolm (21). High-pressure size exclusion chromatography was used to measure both number- and weight-averaged molecular weights. Instrumentation comprised of a Waters 510 solvent pump, a Waters 486 variable wavelength detector, and a Rheodyne rotary injector valve equipped with a 20-μL sample loop (Waters Associates, Milford, MA). A Waters Protein-Pak 125 modified silica column was employed for this study, which has a *general* molecular weight cutoff of less than 100–1000 based upon the configuration of random coiled macromolecules. The column packing was selected based upon its low residual hydrophobicity and minimal ion-exchange capacity. Fulvic acid and the northern Lake Michigan deep (NLMD) porewater eluents were detected at a wavelength of 224 nm.

The selection of the proper standards to characterize the molecular weights of humic substances is in large part determined by their hypothesized structure. A number of investigators (16, 22, 23) have shown that globular proteins standards tend to overpredict the molecular weights of humic substances by a factor of 5 or more and have advocated the use of random coil standards such as polystyrene sulfonates (PSS). While PSS macromolecules are more flexible than many aquatic humic substances, Chin and Gschwend (16) demonstrated that a mobile-phase composition with an ionic strength equivalent to 0.1 M NaCl and a pH of 6.8 can be used with PSS standards because their coiled configuration and that of Suwannee fulvic acid appeared to be nearly identical. Mobile phases used in this study were comprised of Milli Q water buffered with phosphate to a pH of 6.8, and sodium chloride was added to yield an ionic strength of 0.1. The chromatograms were recorded using the Maxima GPC peak integration and molecular weight software. Our standards were comprised of sodium PSS (18K, 8K, 5.4K, 1.8K) and acetone (which also served as our permeation volume probe). The calibration curves were semi-log linear over the range defined by our standards and were used to determine the molecular weight of an analyte, M_i, at some eluted volume i.

Number- (M_n) and weight-averaged (M_w) molecular weights for the humic substances were determined using the following equations:

$$M_n = \sum_{i=1}^{N} h_i / \sum_{i=1}^{N} h_i/M_i \qquad (1)$$

and

$$M_w = \sum_{i=1}^{N} h_i(M_i) / \sum_{i=1}^{N} h_i \qquad (2)$$

where h_i is the height of the sample HPSEC curve eluted at volume i (26). Using the method described by Yau and co-workers (26), we found that dispersion was insignificant in our system, and the chromatograms were evaluated without further need for correction factors.

Organic Carbon and Spectroscopic Analysis. Spectrophotometric analyses of the samples were conducted on a Cary 1 dual-beam scanning UV/VIS spectrophotometer. Samples (identical to those used in the HPSEC analysis) were placed in either a 1- or 10-cm quartz window cuvette and scanned from 700 to 200 nm. We recorded absorbance values at 280 nm (the region where π–π* electron transitions occur for a number of aromatic substances) and at 665 and 465 nm to determine E_4/E_6 ratios. Quantitative ^{13}C-NMR spectra were measured on solutions of approximately 100 mg/mL of the sample dissolved in H_2O–D_2O (1:1) adjusted to pH 7. Measurements were made in 10-mm tubes on a Varian XL 300 spectrometer at 75.429 MHz using inverse gated decoupling with an 8-s delay time. The aromaticity of the analytes was determined by quantifying the peak area in the 110–160 ppm chemical shift band.

Organic carbon data in the aqueous phase were measured using a Shimadzu 5000 TOC analyzer, which is similar to the instrument designed by Sugimura and Suzuki (27). Acidified and purged samples (10–40 μL) were introduced by direct aqueous injection into a platinum-on-alumina catalyst heated to 680 °C in a quartz reaction tube. The carbon analyzer was calibrated with potassium hydrogen phthalate standards at concentrations that ranged from 10 to 40 mg of C/L.

Results and Discussion

Molecular Weight Analysis by HPSEC. All the fulvic acids and Aldrich humic acid eluted from the HPSEC column as a broad, monomodal distribution with subtle shoulders and small subpeaks (Figure 1). The weight- and number-averaged molecular weights and polydispersities of the aquatic fulvic acids measured by HPSEC with polystyrene sulfonate standards are in relatively good agreement with reported values determined by other techniques (Table 1). Molecular weights generated by the HPSEC method are slightly higher than those literature values measured by other methods. This discrepancy is due in part to the use of a UV spectrophotometer as the detector in the HPSEC system. In essence, a number-averaged molar absorptivity (ϵ), based on the whole sample, is used to determine the concentration of material passing through the detector. As will be demonstrated later in this paper, the spectroscopic properties of the dissolved organic carbon vary with molecular weight. The higher molecular weight fractions have greater than the average ϵ and appear to be more abundant than they actually are, while the lower molecular weight fractions (with lower molar absorptivity) appear to be lower in concentration. Nonetheless, similar *trends*
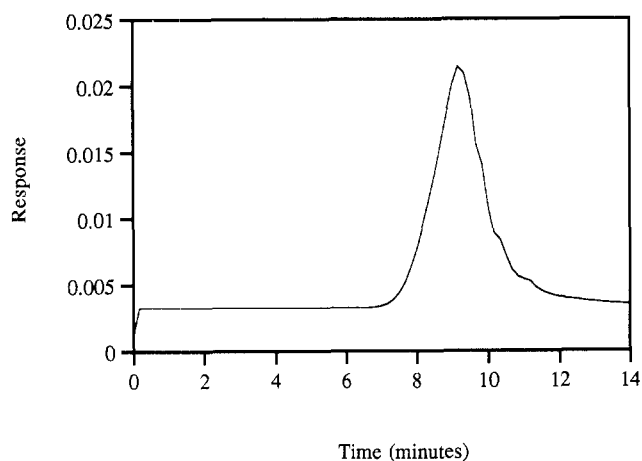
Figure 1. HPSEC chromatogram of Coal Creek fulvic acid.

in the molecular weight distribution of each fulvate sample were observed irrespective of the method used, and these numbers represent for the first time good agreement between HPSEC and other techniques used to measure molecular weights and polydispersity for a large and diverse number of samples. Furthermore, all of these techniques demonstrate that fulvic acids are considerably smaller than other SEC or HPSEC molecular weight distributions reported in the literature (18–20, 22).

Aldrich humic acid was considerably larger than the fulvates, which substantiates observations made by other investigators (16, 23). Our reported humic acid molecular weight is significantly smaller and less polydisperse than previously published values however (20, 29). For instance, the weight-averaged molecular weights reported by Beckett and co-workers (23) using field flow fractionation (FFF) are considerably larger than our HPSEC values. The lack of pretreating the humic materials (no mention of specific cleaning procedures was made in the paper) may have skewed the data analysis toward larger M_w if ash particles were inadvertently introduced into their FFF channel.

The inability of our humic substances to separate into components defined by distinct molecular fractions reflects the fact that they are complex mixtures of high to low molecular weight species that are difficult to resolve chromatographically. Nonetheless, the weight-averaged to number-averaged molecular weight ratio (i.e., the analyte's polydispersity) appears consistent with the hypothesis that these substances occupy a relatively narrower size fraction and do not possess molecular weights that vary by orders of magnitude (16, 21, 23).

Spectroscopic Properties. All humic samples exhibited a featureless increase in absorbance with decreasing wavelength. From our spectra, we measured molar absorptivities of the humic substances and the porewater samples at 280 nm. This wavelength was chosen because $\pi-\pi^*$ electron transitions occur in this region of the UV range (i.e., from ~270 to 280 nm) for phenolic substances, aniline derivatives, benzoic acids, polyenes, and polycyclic aromatic hydrocarbons (30, 31). Since many of these substances are precursors or components of certain types of humic substances (particularly those derived from terrestrial sources), molar absorptivities may yield important clues regarding the degree of aromaticity, source functions, extent of humification, and possibly molecular weight. All of these properties may influence ultimately the behavior in which humic materials can interact with nonpolar organic pollutants, metals, and radionuclides.

Finally, nitrate (a UV-absorbing nutrient ubiquitous to natural waters) does not absorb radiation at 280 nm and would not interfere in the analysis.

The measured molar absorptivities of our samples varied from less than 100 up to approximately 1000 L (mol of OC)$^{-1}$ cm^{-1} and appeared to fall within the range reported by others (Table 1) (32, 33). The commercial humic acid yielded the highest values and is possibly indicative of both its source material and the degree of humification. The molar absorptivities of the aquatic fulvic acid samples ranged from 100 to more than 400 L (mol of OC)$^{-1}$ cm^{-1}. Aquatic fulvic acids are made up of biogenic material from both autochthonous (organic matter derived from primary production in the water column) and allocthonous sources (organic material from terrestrial inputs) (34). The magnitude of ϵ is indicative of both the degree of humification that has occurred and the contribution of terrestrial materials present in the organic matter sources. For example, the Lake Fryxell fulvic acid was extracted from a lake in Antarctica where there are no sources of organic matter associated with higher plants. The organic matter in the lake is derived almost entirely from the algae and bacteria in the lake (35). The relatively low molar absorptivity for this sample is representative of humic materials that were originally formed from the extracellular and/or decompositional byproducts of phytoplankton and bacteria (36, 37). Conversely, the Suwannee River aquatic fulvic acid is comprised of organic precursors from higher plants and has a considerably larger molar absorptivity.

In this study, trends were observed between the spectroscopic properties and structural characteristics of the humic substances. A weak relationship exists between the E_4/E_6 ratio (an indicator of humification) (12, 38, 39) and aromaticity, while a much better correlation was observed between the aromaticity of the fulvic acids and ϵ at 280 nm (Figure 2):

$$\text{aromaticity} = 0.05\epsilon + 6.74 \qquad r^2 = 0.90 \qquad (3)$$

This equation is similar to the one reported by Traina and co-workers (30), but we believe that molar absorptivity based on a mole organic carbon basis rather than on weight of humic acid per unit volume water relationship is less subject to analytical errors, because the ash content of the humic material, if not thoroughly removed by pretreatment, can yield erroneous molar absorptivities. The poorer agreement between aromaticity and the E_4/E_6 ratio can be partially explained by the analytical difficulties in making reliable absorbance measurements at the longer of the two wavelengths (665 nm) for our samples.

A useful semi-predictive relationship was also observed between the molar absorptivity of the humic substances and HPSEC weight-averaged molecular weight (Figure 3). The regression analysis of the data resulted in the following equation for characterizing the relationship between molecular weight and ϵ (280 nm):

$$M_w = 3.99\epsilon + 490 \qquad r^2 = 0.97 \qquad (4)$$

This correlation suggests that the relative amount of aromatic moieties in aquatic fulvic acid increases with increasing molecular weight. Most aquatic organic matter (humic and other materials) from natural waters would be expected to possess molar absorptivities and molecular weights within the confines of this relationship, and eq 4 can be used judiciously to provide ballpark molecular

Table 1. Molecular Weight, Polydispersity, Light Absorbance Properties, and Percent Aromaticity of Humic Substances and Dissolved Organic Carbon in Whole-Water Samples[a]

sample	weight-averaged molecular weight[b]	number-averaged molecular weight[b]	M_w/M_n	reported molecular weights[c]	ϵ (L (mole of OC)$^{-1}$ cm^{-1})	E_4/E_6	aromaticity (%)
Missouri River FA	1460	839	1.7	640 (21)	247	13.9	20.4
Yakima River FA	1560	800	1.9	650 (45)	323	9.47	24.2
Ohio River FA	1330	705	1.9	618 (21)	274	17.2	24.3
Minnesota groundwater FA	1000	639	1.6	650	122	3.53	12.6
Suwannee River FA	2310	1360	1.7	829 (21) 1150[d] (23)	389	20.7	24.8
Coal Creek FA	2230	1180	1.9	743 (21)	401	20.7	27.4
Aldrich HA	4100	1630	2.5	3070[d] (23) 14500 (M_w)[d] (23)	919	7.59	nd
Lake Fryxell	1080	713	1.5	490	150	nd	13
Lake Michigan porewater (3.5 cm)	845 (728)[e]	545	1.6	na	59.6	nd	nd
Lake Michigan porewater (6.5 cm)	1120 (943)[e]	765	1.5	na	113	nd	nd
Lake Michigan porewater (8.5 cm)	1100 (1020)[e]	736	1.5	na	133	nd	nd
Suwannee River water	2190 (2520)[e]	1330	1.6	na	509	nd	nd

[a] Abbreviations: na = not available; nd = not determined. [b] Determined by HPSEC. [c] All data are M_n determined by VPO unless otherwise noted. The references are shown in parentheses. [d] Data obtained using FFF. [e] Estimated from molar absorptivity and eq 4.
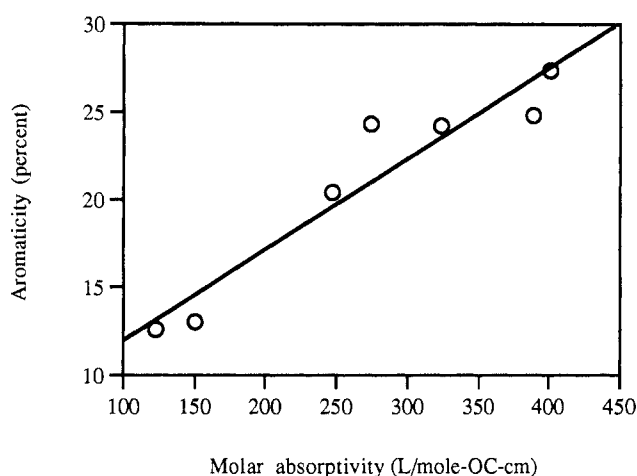

Figure 2. Relationship between the the fulvic acid molar absorptivities at 280 nm and percent aromaticity.
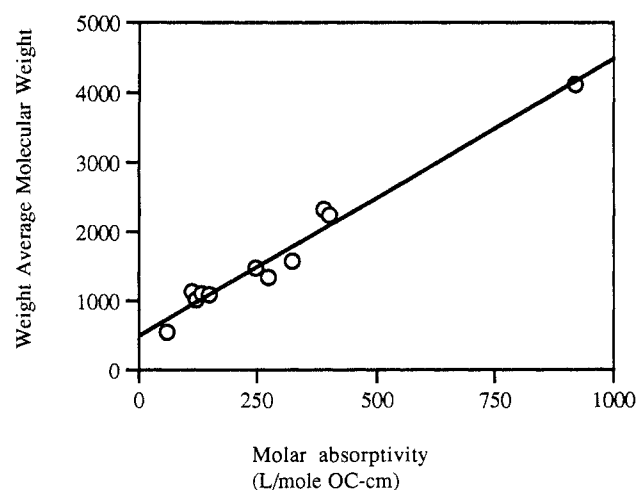

Figure 3. Correlation between molecular weight and the humic substances' molar absorptivities at 280 nm.

weight estimates of organic materials in natural aquatic systems in the absence of other interfering chemical substances (e.g., metal oxides).

A number of investigators (5, 6, 40, 41) have reported a strong correlation between the structure of humic substances and their propensity to bind (complex) organic and inorganic pollutants. For example, those humic substances that appear to be more "aromatic" in structure (as determined by NMR or elemental ratios) were able to strongly bind nonpolar organic compounds (5, 40). In addition, molecular weight and degree of aromaticity of aquatic humic substances have been shown to be important properties that control the amounts of chlorinated disinfection byproducts generated in water treatment (42). Given the strong relationship between ϵ at λ = 280 nm and the degree of aromaticity and molecular weights of aquatic fulvic acids, the molar absorptivity of whole-water samples may be used to a first approximation as a spectroscopic predictor of the chemical reactivity of aquatic humic substances present in the sample.

The application of spectroscopic measurements to estimate the reactivity of humic substances does have limitations. For instance, Chin and Gschwend (41) observed that pore fluid organic matter from a site in Boston Harbor contaminated by petroleum wastes exhibited typical molecular weights and molar absorptivities for marine organic matter (typically 1000–2000 Da and 100–200 L (mol of OC)$^{-1}$ cm^{-1}, respectively) but were nonetheless able to strongly bind pyrene and phenanthrene. While further studies are needed to refine the spectroscopic and chromatographic results of our analysis, this approach can be used to provide *first* estimates of humic substance reactivity from a variety of aquatic sources.

Suwannee River Water and Lake Michigan Porewater Organic Matter Analyses. Finally, the molar absorptivities and molecular weight distributions of organic matter in whole-water samples from the Suwannee River and Lake Michigan porewaters were determined. The Suwannee River water HPSEC chromatogram resembled the reconstituted Suwannee fulvic acid molecular weight distribution (Figure 4a). Actual number- and weight-averaged molecular weights for the whole-water sample were slightly smaller than the isolated fulvic acid fraction (Table 1). These observations suggest that the organic matter in the Suwannee River is comprised of mostly larger amorphous organic molecules and is consistent with the dissolved organic carbon fractionation results for the Suwannee River reported by Aiken and others (45), who found that aquatic fulvic acid and the
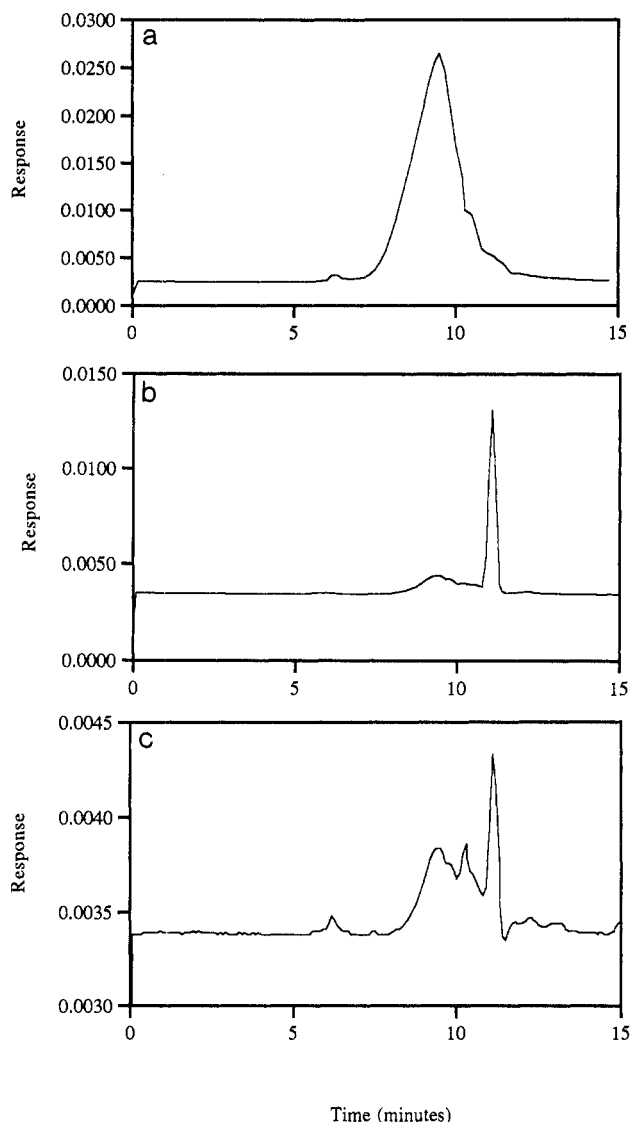
Figure 4. HPSEC chromatograms of (a) unfractionated Suwannee River water, (b) Lake Michigan porewater organic matter from the oxic sediment layer, and (c) Lake Michigan anoxic sediment layer porewater organic matter.
c2.

hydrophilic acid fraction (slightly more hydrophilic, humic-like organic matter) accounted for a total of 84% of the dissolved organic carbon in the Suwannee River. Based on these data, one would expect the organic matter in the whole Suwannee river water to be capable of binding nonpolar organic pollutants in nearly the same capacity as the isolated fulvic acid fraction. Evidence of this can be found in the work of Chiou and co-workers (5) where they reported binding equilibrium constants for p,p'-DDT and 2,4,4'-PCB in whole Suwannee River water and Suwannee fulvic acid to be nearly identical.

The porewater organic matter (PWOM) varied in ϵ from less than 100 to over 130 L (mol of OC)$^{-1}$ cm^{-1} (Table 1). All PWOM assayed from the oxic portion of the core yielded low molar absorptivities, while organic matter from the reduced regions of the core had larger values. The samples were taken from a site located in the northern basin of Lake Michigan, where the organic matter accumulating in the sediments is thought to be derived predominantly from autochthonous sources. This hypothesis is supported by the relatively good agreement between Lake Fryxell fulvic acid sample molar absorp-

tivities and those determined for the lake sediment porewater samples (Table 1).

Porewater organic matter molecular weights were measured from three locations in the northern Lake Michigan core (Table 1). The porewater organic matter exhibited both monomodal and multimodal distributions (Figure 4b,c). Because our porewater samples did not undergo any pretreatment, we interpreted the multimodal distribution observed for the Lake Michigan porewater samples as indicative of the presence of both humic substances and that pool of organic matter which is significantly smaller (i.e., hydrophilic organic acids, etc.). Presumably, the broad first peak is indicative of the more nonlabile fulvic and humic acids, while the later peak is believed to be comprised of smaller dissolved organic species. Porewater organic matter from the upper sediments appeared to be comprised of primarily smaller organic molecules (as shown by the relatively larger second peak in Figure 4b), while deeper regions of the core (below the redox boundary) exhibited a much larger fulvic-like peak (Figure 4c).

The number- and weight-averaged molecular weights of the porewater organic matter from below the redox boundary were significantly higher than the pore fluid organic materials from the oxic region of the core. Moreover, weight-averaged molecular weights estimated from eq 4 and the porewater sample molar absorptivities appear to support this trend (Table 1). It appears that organic matter from reduced porewaters are larger in size and comprised of more nonlabile humic-like substances, while organic material from oxic porewaters contain a significant population of smaller molecules. It is plausible that this segregation of pore fluid organic matter into small and large molecules above and below the redox boundary, respectively, is indicative of selective preservation of the organic materials, possibly coupled with abiotic and biotically mediated condensation reactions (43). This observation is consistent with the findings of others (16, 43, 44) for marine sedimentary porewaters, where the nearly complete depletion of larger porewater organic molecules (measured by UF) near the sediment/water interface (above the redox boundary) was observed. These same investigators found that macromolecular organic carbon primarily resided in the reducing regions of the sediment core.

It is significant that material larger than the fulvic acid isolate is absent from both the Suwannee River water and the Lake Michigan porewater samples. These data suggest that the much of the dissolved organic matter in natural waters does not contain molecular weight fractions larger than aquatic fulvic acid and that the range of molecular sizes (i.e., polydispersities) is relatively narrow.

Conclusions and Implications. Molecular weights of humic substances measured by HPSEC under appropriate conditions were found to yield values that are in general agreement with molecular weights determined by established methods such as VPO and FFF. HPSEC requires relatively small sample volumes and can be used with many samples without preconcentration, allowing molecular size and weight determinations to be made on whole-water samples. The results of these analyses indicate that aquatic humic substances are smaller and less polydisperse than previously believed. The limited measurements on whole-water samples confirm these findings and suggest that the molecular size distribution

of dissolved organic carbon, as a whole, is constrained within a relatively narrow range. These findings have significance for understanding the nature of chemical interactions at the molecular level between dissolved organic carbon and other organic constituents, such as hydrophobic organic compounds.

Combined spectroscopic and molecular weight measurements have yielded important information regarding the bulk properties of aquatic humic materials from diverse sources. In general, the molar absorptivity at $\lambda = 280$ nm of the aquatic humic substances used in this study was found to correlate well with both the molecular weights and the percent aromaticity of these samples. Using these correlations, it is possible, to a first approximation, to estimate the molecular weight and aromaticity of aquatic organic matter from the molar absorptivity (at $\lambda = 280$ nm) on the whole-water sample. Given that both molecular weight and aromaticity have been shown to be important properties in determining the reactivity of dissolved organic carbon in processes such as the facilitated transport of hydrophobic organic compounds and the generation of disinfection byproducts, data on the bulk water sample, such as molar absorptivity and the organic carbon concentration may prove to be useful estimators of the reactivity of a water sample with respect to these processes.

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